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Request for grant of a patent

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1.	Your reference	RVCW / P27101GB
2.	Patent application number *(The Patent Office will fill in this part)*	0220841.1
		10SEP02 E746684-1 D02866 P01/7700 0.00-0220841.1
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	**Royal Veterinary College** University of London Royal College Street London NW1 0TU United Kingdom
		07 SEP 2002
	Patents ADP number *(if you know it)*	7511044001
	If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom
4.	Title of the invention	THERAPY
5.	Name of your agent *(if you have one)*	**ERIC POTTER CLARKSON** **PARK VIEW HOUSE** **58 THE ROPEWALK** **NOTTINGHAM** **NG1 5DD**
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	
	Patents ADP number *(if you know it)*	**1305010**

6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day / month / year)*

8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*	YES

a) *any applicant named in part 3 is not an inventor; or*
b) *there is an inventor who is not named as an applicant, or*
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Description	6
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	NO
Request for preliminary examination and search *(Patents Form 9/77)*	NO
Request for substantive examination *(Patents Form 10/77)*	NO
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature E Potter Clarkson Date
ERIC POTTER CLARKSON 6 September 2002

12. Name and daytime telephone number of person to contact in the United Kingdom **0115 9552211**

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THERAPY

The present invention relates to

Protocol for Mesenchymal stem cell therapy of tendon injuries in the horse

Criteria for inclusion of cases:
Superficial digital flexor tendon or suspensory ligament injury of the palmar aspect of the metacarpus which does not involve a tendon sheath. Only lesions with defined core lesions will be included and the current injury should be more than 3 weeks and less than 3 months in duration.

Protocol:

1) Baseline clinical examination to include full ultrasonographic examination and blood sample (for preparation of platelet-rich plasma and markers studies).

2) Cross-sectional areas of the damaged tendon to be calculated including tendon and lesion cross-sectional area for all seven transverse levels in the metacarpal region to give a percentage involvement of the lesion (severity).

3) After sedation (alpha 2 agonist and butorphanol), clipping and scrubbing over the sternum, individual sternebrae will be identified using diagnostic ultrasound and the inter-sternebral space marked on the skin with a sterile marker

4) Local infiltration of local anaesthetic will be placed over the site for marrow aspiration (in centre of two adjacent sternebrae). A stab incision is made through the skin using a no. 11 scalpel. A Jamshidi biopsy needle is introduced until it hits to the sternebra. It is pushed a further 3-4cm into the sternebra and then 2x2ml aliquots of bone marrow from each of two sternebrae is aspirated into 2ml syringes, pre-loaded with 500iu (0.2ml of 5000iu/ml in each syringe) heparin.

5) After the aliquots have been obtained, a further 20ml is withdrawn from one sternebra into a syringes pre-loaded with the same concentration of heparin (2ml in 20ml syringe). The bone marrow aspirate is then spun down at 2000rpm for 20 mins and the supernatant collected, transferred to sterile 20ml tubes, and frozen at –20C.

6) 2ml aliquots transferred into sterile 5ml tubes

7) Immediate transfer to Stanmore on ice.

8) Aliquots used for recovery and culturing of MSCs (see attached protocol on page 4)

9) Expansion of MSCs over approximately 1-2 week period until colonies of MSCs formed on plastic. Cella passaged and expanded further (for ~5 days until confluent) when there are approximately 7 x10^6 cells/ml.

10) Cells removed from the flasks and divided into 3 aliquots.

11) Spun down in sterile tubes (1000rpm for 10 minutes) to pellet the cells.

12) Aliquot 1 - used to characterize cells (i.e. ensure they are MSCs).
Aliquot 2 - cells frozen down in DMSO (for potential future use)
Aliquot 3 - prepared for injection:

13) Supernatant removed

14) Cell pellet (approximately 7x x10^6 cells) washed with fresh DMEMs without serum

15) Spun down in sterile tubes (1000rpm for 10 minutes) to pellet the cells

16) Cells resuspended in 2ml platelet-rich plasma (PRP) (or marrow supernatant), previously thawed, derived from the same horse

17) Cells injected into the damaged tendon in a sterile fashion under sedation and perineural analgesia using multiple needle stabs (23G, 1 inch needle – 10 injections of 0.1ml along the length of the lesion

18) Limb bandaged with a standard modified Robert Jones bandage

19) Tendons scanned at 3 days after injection and then the horse discharged from the hospital.

20) Limb to remain bandaged for 2 weeks and then stable bandaged

21) Horse is box-rested for 1 week and then given walking in hand exercise for a further 3 weeks before repeat ultrasound examination.

22) Repeat ultrasound examinations and blood samples at monthly intervals while following the controlled exercise programme shown below:

GUIDELINES FOR CONTROLLED EXERCISE PROGRAMME

Level	Minimum weeks after injury	Duration and nature of exercise
1	**0 – 8**	**30 minutes walking daily building this up to 45 minutes**
2	**9 - 32**	**walking + 5 minutes trotting building up to 30 minutes**
	9 - 12	40 minutes walking and 5 minutes trotting daily
	13-16	35 minutes walking and 10 minutes trotting daily
	17-24	30 minutes walking and 15 minutes trotting daily
	25-28	25 minutes walking and 20 minutes trotting daily
	29-32	15 minutes walking and 30 minutes trotting daily
3	**33 - 52**	**Walk and trot with restricted canter work**
	33-36	45 minutes exercise daily with slow canter up to 1 mile twice weekly
	37-40	45 minutes exercise daily with slow canter up to 1.5 mile twice weekly
	41-44	45 minutes exercise daily with one 3 furlong gallop three times a week
	45-48	45 minutes exercise daily with one 6 furlong gallop three times a week
	49-52	Increase exercise level gradually to full race/competition training
4	**From 52 weeks**	**Full race/competition training**

23) Compare results with an age-matched group of horses with similar lesions managed conservatively with the above exercise programme alone.

Outcome measures:

Ultrasonographic progression
Marker levels
Athletic outcome

If euthanased, tendon recovered for mechanical and matrix analyses.

Protocol for equine mesenchymal stem cell isolation from bone marrow

Materials

Ficoll	Marrow
5ml syringe	pipette 12ml x3
Green syringe needle	pipettor
Universal	waste pot
Transfer pipette x2	

FICOLL GRADIENT

1. Invert bottle of Ficoll to mix, snap off polypropylene cap, insert syringe through septum injecting air to equalise pressure. Invert bottle and withdraw 3ml liquid.
2. Gently lay 4ml bone marrow onto 3ml Ficoll. The two layers are best achieved by holding the universal straight up and dispensing the marrow slowly down the side of the universal.
3. Centrifuge at 1510rpm for 30 minutes (program 5 centrifuge in room 2 stops slowly and does not disturb layers) so that a straw coloured buffy layer forms in between the plasma and Ficoll erythrocyte residue.
4. Remove buffy layer to a fresh universal using a transfer pipette. Only mononuclear sells should be left in suspension

SEEDING FLASKS

Materials

DMEM: 500ml 4500mg/L glucose, L-glutamine and sodium pyruvate
Foetal calf serum 10%, 50ml
Penecillin 50 u/ml and Streptomycin 50µg/ml

T75 x2/T25 x2
Waste pot
23 needle
5ml syringe
12ml pipettes x3

5. Wash the buffy layer by resuspending the cells in 10ml DMEM. Spin at 1500rpm for 10 minutes to remove heparin and Ficoll.
6. Remove supernatant. Stem cells should be in the pellet.
7. Resuspend pellet in 2ml DMEM using a 23 guage syringe needle to give a single cell suspension.
8. Divide cells into two T75 flasks. T25s can be used if there was only a small volume of aspirate taken.

WASHING CELLS

9. Allow primary seeded cells to adhere to the flask for two days before changing the medium. (If setting up cells on Thursday, leave over the weekend).
10. Change medium every two days.

OBSERVATIONS

-The flasks may appear cloudy. This is because there are erythrocytes in the suspension that will be washed off in subsequent DMEM washes.

-Stem cells can initially be observed as round shiny objects that have adhered to the flask unlike the surrounding cells in suspension.

-CFU-Fs should be seen after two weeks in culture.

N.B. 100-500 Human MSCs result from 50-100 million cells introduced into culture. (Hayensworth S.E. et al)

Horse stem cells
07/06/02

Aim

Isolation and expansion of horse MSCs with a view to reinjecting the cells into the tendon.

Hypothesis

The number of cells in the initial 4ml of aspirate extracted from horse marrow will yield a larger number of cells compared to the final 4ml.

1. Marrow aspirate was taken from the horse sternum in the following aliquots (500u/ml of heparin was used):

    1) Sample 1: 1-2ml
    2) Sample 2: 3-4ml
    3) Sample: 5-6ml (given to horse)
    4) Sample 3: 7-8ml
    5) Sample 4: 9-10ml

2. Sample 1 and 2 were combined to give the first 4ml of a 10ml sample. Sample 3 and 4 were combined to give the final 4ml of the 10ml sample. They were named:

- HS1 A : Sample 1+2
- HS1 B : Sample 3+4

3. The technique for isolating stem cells from bone marrow outlined above was followed. Cells were passaged at into 2x T75 flasks and were left in culture for 19 days.
4. Cell were counted and passaged into 2x T175 flasks.

Cell count:

HSA 1 P0 T75 .....3.1 x 10^6 cells/ml
HSB1 P0 T75 ..... 7.2 x 10^5 cells/ml

5. Cells were cultured for a 5 days until they reached confluency. Cells were counted at P1 and frozen down in DMSO.

Cell count:

HSA 1 P1 T175 ..... 7.8 x 10^6 cells/ml
HSB 1 P1 T175 ..... 4.48 x 10^6 cells/ml

Results

Cell count at P0:

HSA 1 P0 T75 .....3.1 x 10^6 cells/ml
HSB1 P0 T75 ..... 7.2 x 10^5 cells/ml

Cell count at P1:

HSA 1 P1 T175 ..... 7.8 x 10^6 cells/ml
HSB 1 P1 T175 ..... 4.48 x 10^6 cells/ml

Conclusion

There is a higher yield of cells in the initial 4ml of aspirate compared to the last 4ml of marrow extracted from the horse. The cells can be expanded in culture and reach confluency within 5 days.

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